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Effects f Lysostaphin and Its Two Active Components on Stable Wall-Defective Forms of *Staphylococcus aureus*

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Previous experiments [1] have shown that stable wall-defective forms of *Staphylococcus aureus* induced by either methicillin or lysostaphin [2, 3] were resistant to antimicrobial agents that act solely on the cell wall and sensitive to only those that partly or solely affect the cell membrane or inhibit protein synthesis. At the same time, it was also shown that lysostaphin inhibited the growth of both types of wall-defective forms of *S. aureus* [1].

The only known activity of lysostaphin is lysis of the staphylococcal cell wall by the action of one of its 2 active components, lytic peptidase. This enzyme catalyzes a hydrolytic reaction that liberates N-terminal glycine and alanine from the staphylococcal cell wall [4]. The other active component, hexosaminidase, cleaves the glucosaminyl-muramic acid bond of the solubilized staphylococcal cell wall peptidoglycan but does not lyse the intact cell wall of viable staphylococci [4]. Lytic peptidase and hexosaminidase comprise approximately 65% and 5% of lysostaphin by weight, respectively, and the remaining 30% of lysostaphin by weight contains the nonlytic peptidase (unpublished data).

It is the purpose of this communication to report a series of experiments performed in order to understand better the action of lysostaphin, lytic peptidase, and hexosaminidase on stable wall-defective forms of *S. aureus* induced by methicillin and lysostaphin.

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Materials and Methods

Culture media. Brain heart infusion broth (Difco) was used with salts added to final concentrations of 5% NaCl and 0.2% $MgSO_4$. The *p*H of this medium was 7.0. Each hypertonic agar plate contained 10 ml of 1.05% Noble agar with 5% heat-inactivated human serum and hypertonic brain heart infusion broth [5].

Stable wall-defective forms of S. aureus. Stable wall-defective forms of the August Harmon (AH24H) strain of *S. aureus,* phage type 6/7/42D/54/75, originally recovered from a patient with endocarditis, were used in these experiments. The methods of induction and propagation of stable wall-defective forms of the AH24H strain of *S. aureus* induced by methicillin [6] and lysostaphin [5] have been described previously. The former had been transferred more than 100 times and the latter 50 times in the absence of the respective inducing agents and had remained stable.

Lysostaphin and its 2 active components. Partially purified lysostaphin lot #ML 0 138 with a lytic potency of 165 units/mg was used in these studies. Lytic peptidase (primarily a glycinase) was isolated from lysostaphin by Sephadex gel filtration and shown to be free of hexosaminidase activity. Similarly, the hexosaminidase (an exo-glucosaminidase) was obtained from lysostaphin free from contaminating lytic peptidase by Sepha-

dex gel filtration. The enzymes were dissolved in sterile water that had been adjusted to *p*H 4.8 with glacial acetic acid and stored at 4 C.

Methods for susceptibility testing. Two methods were used in studying the effects of the enzymes on the stable wall-defective forms of *S. aureus*. The first was a serial 2-fold broth dilution test to determine the minimal inhibitory concentrations (MIC) [1]. The second method was a viable cell count of the wall-defective *S. aureus* after they had been exposed to the enzymes. The enzyme to be tested was added to a 1:100 dilution of an overnight culture of wall-defective forms of *S. aureus* to make the desired final concentration. A similar broth culture without the enzyme was set up as a control. The cultures were incubated in a 37 C water bath. Colony counts for viable colony-forming units before and at intervals after the wall-defective staphylococci had been exposed to the enzyme were determined by plating on hypertonic agar plates. A sterile L-shaped glass rod was used to spread 0.1 ml of inoculum over the surface of the plate. Plates were then sealed with cellophane tape and incubated at 37 C for 5 days before colonies were counted.

Method for determination of hexose and hexosamine contents of membranes of wall-defective forms of S. aureus. Membranes were prepared by lysing the wall-defective *S. aureus* in cold 0.005 M phosphate buffer (*p*H 6.0). Membranes were recovered from the lysate by centrifugation. Nucleic acids adhering to the membranes were removed by treatment with desoxyribonuclease in 0.02 M phosphate buffer (*p*H 7.0) containing 5×10^{-4} M magnesium ion. The membranes were washed further with IM potassium chloride and distilled water prior to lyophilization.

Membranes for carbohydrate determinations were hydrolyzed in constantly boiling hydrochloric acid in sealed tubes placed in an oil bath at 95 C for 40 min. Hydrochloric acid was removed from the hydrolysate under vacuum on a rotary evaporator, with the last traces of acid being removed by overnight storage in a vacuum desiccator over solid potassium hydroxide. Reducing sugars

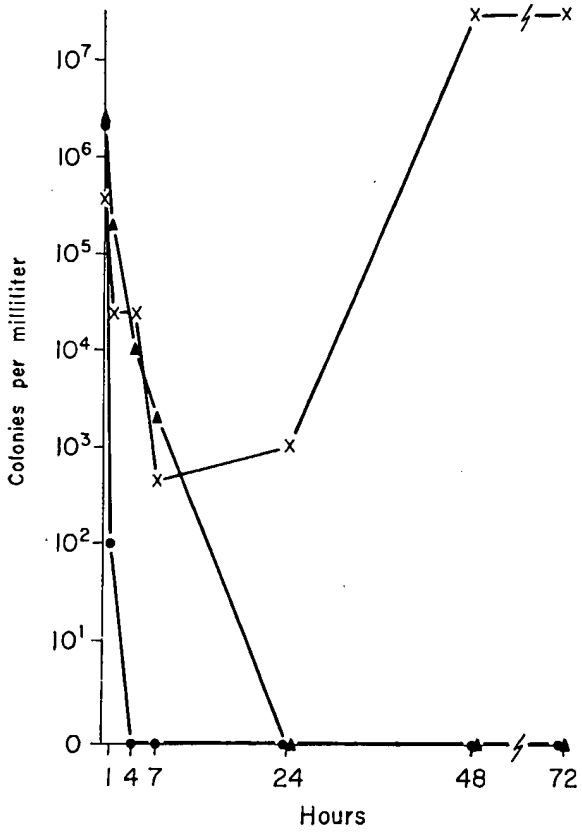

Figure 1. The effect of different concentrations of lysostaphin on stable wall-defective form of *S. aureus* induced by methicillin. Lysostaphin 480 μg/ml (circle), 100 μg/ml (triangle), and 50 μg/ml (cross).

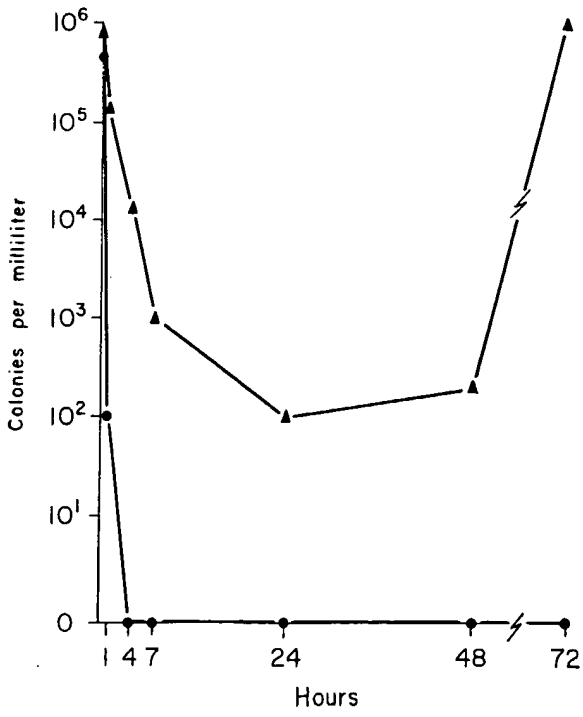

Figure 2. The effect of different concentrations of lysostaphin on stable wall-defective form of *S. aureus* induced by lysostaphin. Lysostaphin 480 μg/ml (circle) and 100 μg/ml (triangle).

Table 1. Minimal inhibitory concentrations of lysostaphin and its 2 active components for stable wall-defective forms of *S. aureus*

	Wall-defective forms of *S. aureus*	
	Lysostaphin-induced	Methicillin-induced
Lysostaphin	46.8*	46.8
Lytic peptidase ..	100	200
Hexosaminidase ..	6.25	12.5

* Microgram per ml.

were determined by the method of Park and Johnson [7], and hexosamine content by the procedures of Spiro [8, p. 56].

Results

Effects of lysostaphin and its 2 active components on stable wall-defective forms of S. aureus. Figures 1 and 2 illustrate the effects of different concentrations of lysostaphin on stable wall-defective forms of *S. aureus* induced by methicillin and lysostaphin, respectively. High concentrations of lysostaphin sterilized cultures of both types of wall-defective staphylococci in a relatively short period of time, while lower concentrations lowered the viable count significantly but only temporarily.

Table 1 shows the MIC of lysotaphin, lytic peptidase, and hexosaminidase for the lysostaphin- and methicillin-induced stable wall-defective forms of *S. aureus*. The MIC of lytic peptidase was slightly higher than that of lysostaphin, while the MIC of hexosaminidase was significantly lower. Figures 3 and 4 illustrate the effects of lysostaphin, lytic peptidase, and hexosaminidase on stable wall-defective forms of *S. aureus* induced by methicillin and lysostaphin, respectively. At the same concentration, hexosaminidase is the most

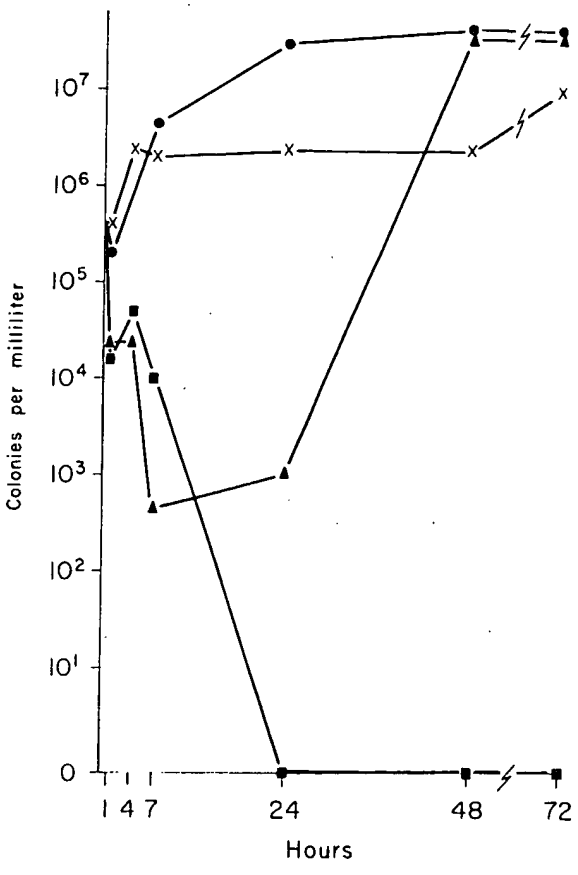

Figure 3. Comparison of the effect of 50 μg/ml of lysostaphin, lytic peptidase, and hexosaminidase on stable wall-defective form of *S. aureus* induced by methicillin. Control (circle), lysostaphin (triangle), lytic peptidase (cross), and hexosaminidase (square).

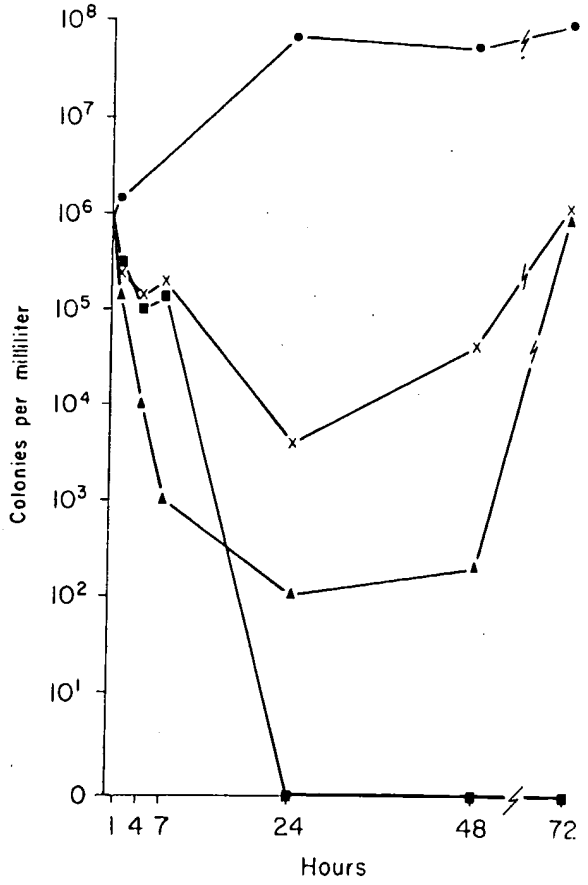

Figure 4. Comparison of the effect of 100 μg/ml of lysostaphin, lytic peptidase, and hexosaminidase on stable wall-defective form of *S. aureus* induced by lysostaphin. Control (circle), lysostaphin (triangle), lytic peptidase (cross), and hexosaminidase (square).

Table 2. Reducing and amino-sugar contents of membranes of wall-defective forms of *S. aureus*

Membrane	Reducing sugar (μg glucose equiv/mg membrane)	Amino sugars (μmoles/mg membrane)
Methicillin-induced	25	0.0099
Lysostaphin-induced	29	0.0099

effective in killing the wall-defective staphylococci while lysostaphin is slightly more active than lytic peptidase.

Hexose and hexosamine contents of membranes of wall-defective forms of S. aureus. The results presented in table 2 show reducing sugar levels of 25 μg and 29 μg glucose equivalents per ml of membrane for methicillin- and lysostaphin-induced wall-defective forms of *S. aureus,* respectively. They are equivalent to 0.12 μmole and 0.16 μmole of glucose, respectively. These values are several orders of magnitude greater than the amounts of amino sugar found and suggest that the bulk of the carbohydrate associated with the membrane is not an amino sugar.

Discussion

The results of these experiments confirm previous observations that stable wall-defective forms of *S. aureus* are susceptible to lysostaphin [1]. The hexosaminidase component of lysostaphin appears to be responsible for the principal activity of lysostaphin on the wall-defective staphylococci, though the lytic peptidase component also had some lethal effect. It has been shown that lysostaphin is capable of lysing isolated fragments of cell walls of *S. aureus* [2]. The possibility existed that fragments of cell wall remained attached to the membranes of these wall-defective staphylococci. The action of lysostaphin may then have been on these fragments of cell wall, should they indeed exist. However, the levels of hexosamine found in the membranes were exceedingly low (less than 0.01 μmole/mg of membrane). The hexosamine content of the *S. aureus* cell wall is more than 1 μmole/mg of cell wall [9]. From the information we have at hand, it is not possible to rule out conclusively the possibility of minute cell wall fragments (at least the amino sugar moiety) remaining attached to the membrane. The values reported here, 0.0099 μmole/mg of membrane, might be falsely high because of the limitation of the analytic technique used. The values might be lowered by the use of larger samples of membranes for analysis. It is also possible that some hexosamine could well be an integral part of the membrane itself. The hexose contents of the methicillin- and lysostaphin-induced wall-defective forms of *S. aureus* were 2.5% and 2.9%, respectively, comparable to the value of 1.7% (small particle fraction) reported by Salton [10].

Since there is no good evidence that fragments of cell walls are left on the membrane of these wall-defective forms of *S. aureus,* lysostaphin probably has a site of action other than the cell wall to account for its ability to kill wall-defective staphylococci. The site and mechanism of this action is unknown at the present time.

Summary

Stable wall-defective forms of *Staphylococcus aureus* were shown to be susceptible to lysostaphin. The principal action of lysostaphin on these wall-defective staphylococci resulted from the activity of its minor active component, hexosaminidase. It is postulated that lysostaphin has an additional site of action on *S. aureus* over and above its presently known action on the cell wall.

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